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Sulfur mustard induces apoptosis in lung epithelial cells *via* a caspase amplification loop☆,☆☆

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ABSTRACT

Sulfur mustard (SM [bis-(2-chloroethyl) sulfide]) is a chemical warfare agent that causes skin blisters presumably due to DNA alkylation and cross-links. We recently showed that SM also induces apoptotic death in cultured normal human bronchial/tracheal epithelial (NHBE) cells and small airway epithelial cells (SAEC) *in vitro*. In this process, caspases-8 and -3, but not caspase-9, were strongly activated; this suggests a death receptor pathway for apoptosis. We now show that rat lungs were induced to undergo apoptosis *in vivo* following exposure of rats to SM by inhalation. Further study of the mechanism of apoptosis due to SM was performed with cultured NHBE cells and SAEC using tetrapeptide inhibitors of caspases-3, and -8. Inhibition of caspase-8 drastically reduced the activation of caspase-3 and almost eliminated that of caspase-9. Moreover, caspase-3 inhibition markedly reduced the activation of caspase-8 and also almost completely inhibited activation of caspase-9. These results suggest a death receptor pathway of apoptosis that utilizes a feedback amplification mechanism involving an activated death receptor complex that leads to the activation of caspase-9 *via* a caspase-3 pathway. These results may be important for the design of inhibitors of these pathways for therapeutic intervention to attenuate SM injury in respiratory tract lesions.

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1. Introduction

Sulfur mustard (bis-(2-chloroethyl) sulfide; SM) is a highly reactive vesicating compound. While originally used as a chemical warfare agent, SM now poses a new threat to both the military and civilians (Saladi et al., 2006). In addition to severe skin blistering, SM causes epithelial damage to eyes and respiratory tract (Balali-Mood and Hefazi, 2006; Dacre and Goldman, 1996). SM injury to any of these target organs may be quite debilitating; however, SM mortality is associated with pulmonary damage and respiratory tract lesions (Urbannetti, 1988). In humans, SM inhalation exposure is associated with laryngitis, tracheobronchitis, bronchiolitis obliterans, bronchopneumonia, chronic obstructive pulmonary disease (COPD), bronchiectasis, asthma, and large airway narrowing; these conditions are exacerbated over time (Balali-Mood and Hefazi, 2006; Ghanei and Harandi, 2007; Bijani and Moghadamnia, 2002; Hefazi et al., 2005; Emad and Emad, 2007). The mechanisms that underlie these SM-induced respiratory lesions, however, are unknown.

SM induces cell death in the basal epidermal layer as well as in the epithelial layers of the lung and cornea (Meier et al., 1984; Gross et al., 1988; Petrali et al., 1990; Smith et al., 1990, 1991; Papirmeister et al., 1991; Ray et al., 2008). Our results and those from other laboratories indicate that cell death due to SM involves apoptosis as an early event; however, also involves an apoptotic to necrotic continuum depending on SM concentration and time after SM exposure (Dabrowska et al., 1996; Rosenthal et al., 1998; Ray et al., 2008). In vertebrates, apoptosis is subdivided into two forms: an extrinsic pathway, mediated by death receptors within the TNFR (tumor necrosis factor receptor) superfamily; and an intrinsic pathway, mediated by the release of apoptogenic factors

Abbreviations: SM, sulfur mustard; NHBE, normal human bronchial/tracheal epithelial; SAEC, small airway epithelial cells.

☆ The opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army or the Department of Defense. The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, Publication No. 85-23, 1996), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

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from the mitochondria (Kumar, 1999). Both forms of apoptosis are mediated by caspases, cysteine proteases that recognize aspartate at their substrate cleavage site (Porter and Janicke, 1999). Their proteolytic substrates are key proteins involved in the structure and integrity of the cell. The extrinsic and intrinsic modes of apoptosis rely primarily on caspases-8 and -9, respectively, as initiator caspases (Kumar, 1999).

The extrinsic pathway begins with the activation of one of the death receptors by either the binding of its cognate ligand or overexpression/inappropriate cross-linking of the death receptor itself or both (Aragane et al., 1998). Following activation, FADD (Fas associated death domain) as well as additional adaptor proteins, are recruited to the cytoplasmic C-terminal death domains of receptors, followed by procaspase-8. Procaspase-8 then undergoes autocatalytic cleavage by induced proximity at the multiprotein death-inducing signaling complex (DISC) and, in turn, proteolytically cleaves and activates effector caspases, caspases-3, -6 and -7.

The intrinsic mode of apoptosis is centered around signaling from the mitochondria; this involves the release of pro-apoptotic factors (e.g., AIF (apoptosis inducing factor), cytochrome c and Smac/Diablo), which depends on the apoptotic stimulus as well as on the balance of members of the Bcl-2 family of proteins (Wong and Puthalakath, 2008). Some of these factors (e.g., Bcl-2 and Bcl-xL) stabilize mitochondria, while other members, including Bax and Bak, destabilize this organelle. Cytochrome c and ATP/dATP bind Apaf1 (apoptosis activating factor 1) in the cytosol to form the heptameric apoptosome, which activates the initiator procaspase-9, which in turn activates the effector caspases.

The extrinsic and intrinsic pathways may not be mutually exclusive. For example, many cells require the cleavage of Bid by caspase-8, which in turn activates Bax and/or Bak and thus the intrinsic pathway and caspase-9, in order to effectively carry out DR-mediated apoptosis (Fulda et al., 2001; Scaffidi et al., 1998). An alternate cross-talk pathway involving the activation of caspase-9 by caspase-3 has also been described (Fujita et al., 2001). Therefore, in deciphering the apoptotic pathways, it is necessary to detect not only which caspases are activated but also the temporal relationships between their activation signals.

Following SM exposure, keratinocytes undergo a combination of both intrinsic and extrinsic apoptosis (Rosenthal et al., 2000, 2003). The intrinsic response involves increased levels and activation of calmodulin upstream to this pathway. SM also increases both the Fas receptor and its ligand, causing procaspase-8 activation. Inhibiting either the calmodulin (Simbulan-Rosenthal et al., 2006) or the DR pathway (Rosenthal et al., 2003) *via* expression of antisense or dominant-negative constructs, respectively, reduced SM toxicity in culture and vesication in human skin grafted onto nude mice. Mol et al. (2009) recently showed that peptide inhibitors specific for caspase-8 or -9 could prevent SM-induced microvesication in human skin in organ culture. In the present study, we investigated whether apoptosis due to SM in airway epithelial cells also involves these two pathways and the cross-talk between the two. We studied the activation of caspases-9, -8, -3, and the effects of inhibiting caspases-8 and -3, by specific peptide inhibitors in cultured human airway epithelial cells.

2. Experimental

2.1. Cells

Frozen stock primary normal human bronchial/tracheal epithelial (NHBE) cells and small airway epithelial cells (SAEC), as well as their growth media (BEGM, basal epithelial growth medium; SAGM, small airway growth medium), were obtained from Lonza, Walkersville, MD. NHBE cells and SAEC were maintained in serum-free BEGM or SAGM, respectively, supplemented with bovine pituitary extract, hEGF, hydrocortisone, epinephrine, transferring, insulin, and retinoic acid. Since NHBE cells and SAEC become irreversibly contact-inhibited, they were subcultured or used when they reached 80% or less confluence. To maintain consistency in results, cells from the same donor were used up to a culture passage 3.

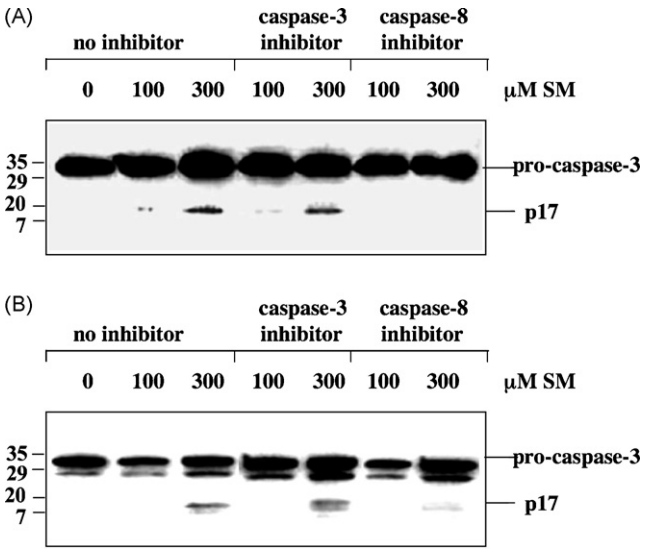

Fig. 1. SM-induced activation of caspase-3 in normal human small airway epithelial cells (SAEC) and bronchial/tracheal epithelial (NHBE) cells. SAEC (A) or NHBE (B) cells were exposed to the indicated doses of SM and cytosolic extracts were derived after 16 h and subjected to immunoblot analysis using antibodies specific for executioner caspase-3. The positions of the immunoreactive proteins are indicated. Results shown are representative of the whole.

For exposure to SM, cells were grown to about 80% confluency and then exposed to SM diluted in BEGM or SAGM to final concentrations of 50 (Figs. 2 and 3), 100 (Figs. 1–3), or 300 μM (Figs. 1–3). SM undergoes rapid hydrolysis in aqueous solution and was, therefore, diluted in respective media immediately before use. Cells were exposed to diluted SM in growth medium as described previously (Ray et al., 1995). Briefly, a formulation consisting of 5 μl of neat SM oily globule frozen in 10 ml of cell growth medium was thawed by warming to room temperature, and vortexed at top speed for 1 min to solubilize the SM in the medium and produce a 4-mM stock solution. The stock solution was then added to the cell culture medium in flasks for indicated SM concentrations, and media was not changed until cells were harvested after 16 h for further analyses.

2.2. Chemicals

SM (bis-(2-chloroethyl) sulfide; >98% purity) was obtained from the US Army Edgewood Chemical Biological Center (ECBC), Aberdeen Proving Ground, MD, USA.

2.3. Fluorometric assay of caspase activity

Cytosolic extracts were derived from pooled floating and attached cells and subjected to fluorometric caspase-3 activity assays using fluorescent tetrapeptide substrate specific for caspases-3 (Ac-DEVD-aminomethylcoumarin (AMC), BioMol, Plymouth Meeting, PA) as previously described (Simbulan-Rosenthal et al., 2002). For the fluorometric caspases-8 and -9 activity assays, the tetrapeptide substrates specific for caspases-8 and -9 (Ac-IETD-AMC and Ac-LEHD-AMC, respectively; BioMol, Plymouth Meeting, PA) were utilized in essentially the same reaction assay conditions as for caspase-3. Free AMC, generated as a result of cleavage of the aspartate-AMC bond, was monitored over 30 min with a Wallac Victor^{3}V fluorometer (Perkin Elmer, Waltham, MA) at excitation and emission wavelengths of 360 and 460 nm, respectively. The emission at 460 nm from each sample was plotted against time, and linear regression analysis was used to determine the initial velocity (slope) for each curve, which yielded the activity. The activity of the samples from the treated (SM alone or SM + inhibitor) cells divided by that of the untreated controls (0 μM SM) gave the fold increase. The results were presented as fold increase due to the marked differences in the inherent levels of caspase activities between the two cell lines, SAEC and NHBE. Inhibitors of caspase-8 (Z-IETD-fmk) and caspase-3 (Z-DEVD-fmk) were obtained from BioMol, Plymouth Meeting, PA, used at a concentration of 50 μM, and added to cells 30 min prior to addition of SM. In the amino acid components of these tetrapeptides, the nitrogen atoms are in the amide form and the acid groups are in the ester form; none of these should be a nucleophile to react with SM.

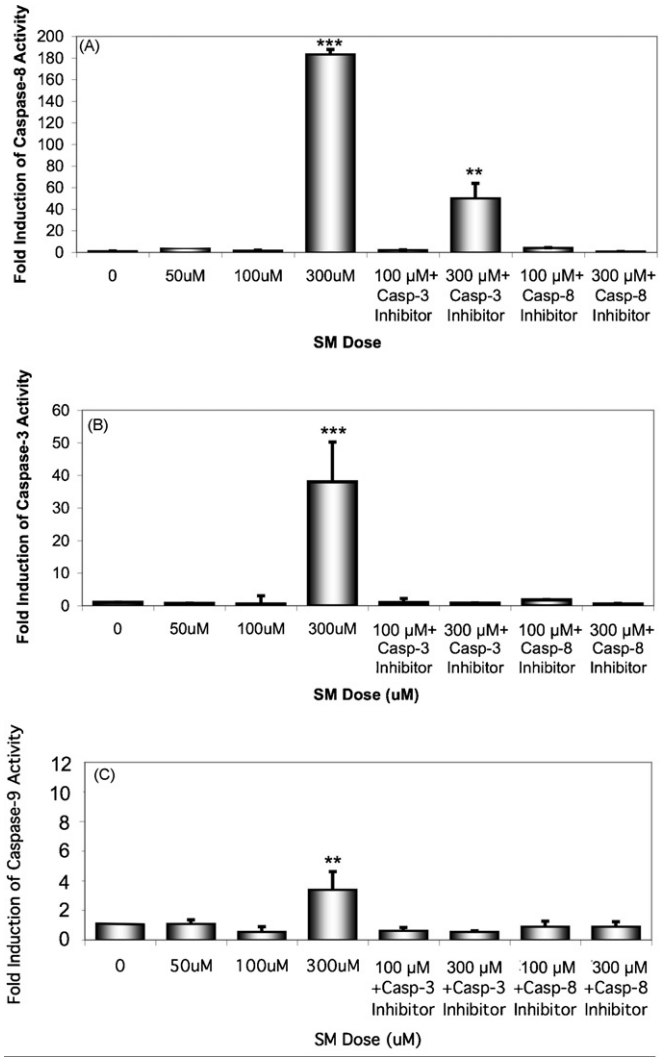

Fig. 2. SM-induced activation of caspases-3, -8, and -9 in normal human small airway epithelial cells. Cells were exposed to the indicated doses of SM, with or without pretreatment with tetrapeptide inhibitors of caspase-3 or -8. Cytosolic extracts were derived after 16 h and subjected to quantitative fluorometric caspase activity assays with Ac-IETD-AMC (for caspase-8) (panel A), Ac-DEVD-AMC (for caspase-3) (panel B), or Ac-LEHD-AMC (for caspase-9) (panel C) as substrates. Baseline (0 μM SM) enzyme activity for each caspase was as follows (in relative fluorescence units/min): 1.01 (caspase-3) and 0.6 (caspases-8 and -9); details are provided in Section 2. Error bars represent average ± SEM, $n = 3$. Significance relates to untreated control (0 μM SM) as follows, $^{**}p < 0.01$, $^{***}p < 0.001$.

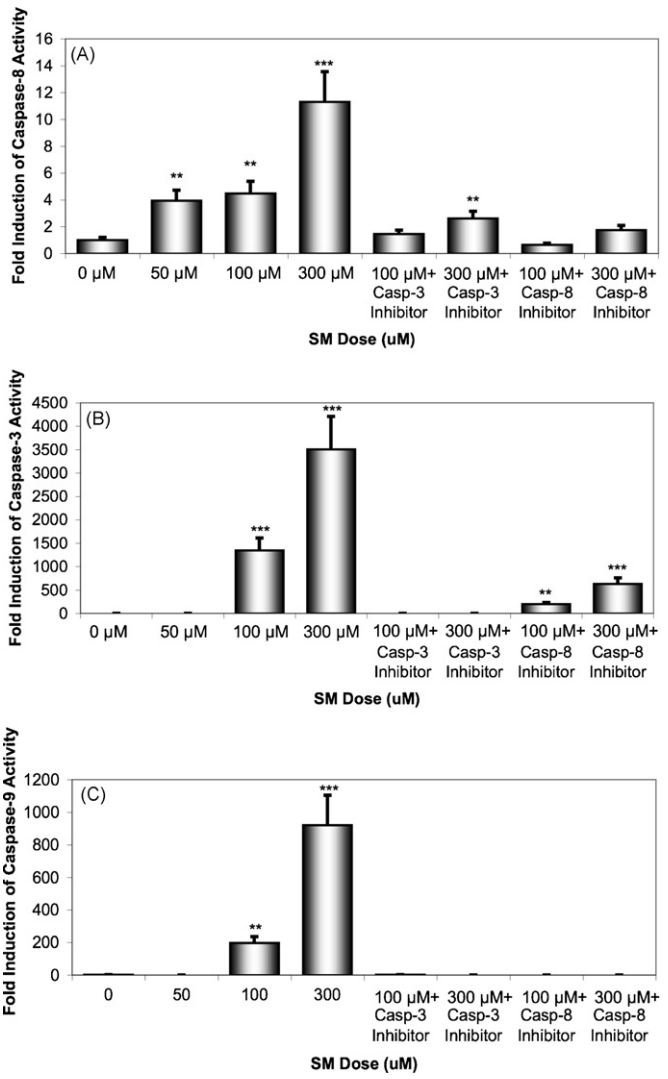

Fig. 3. SM-induced activation of caspases-3, -8, and -9 in normal human bronchial/tracheal epithelial cells. Cells were exposed to the indicated doses of SM, with or without pretreatment with tetrapeptide inhibitors to caspase-3 or -8. Cytosolic extracts were derived after 16 h and subjected to quantitative fluorometric caspase activity assays with Ac-IETD-AMC (for caspase-8) (panel A), Ac-DEVD-AMC (for caspase-3) (panel B), or Ac-LEHD-AMC (for caspase-9) (panel C) as substrates. Baseline (0 μM SM) enzyme activity for each caspase was as follows (in relative fluorescence units/min): 0.48 (caspase-3), 32.8 (caspase-8) and 0.2 (caspase-9); details are provided in Section 2. Error bars represent average ± SEM, $n = 3$. Significance relates to untreated control (0 μM SM) as follows, $^{**}p < 0.01$, $^{***}p < 0.001$.

2.4. Immunoblot analysis

SDS-PAGE and transfer of separated proteins to nitrocellulose membranes were performed according to standard protocols. Proteins were measured (DCA protein assay; BioRad, Hercules, CA) and Ponceau S (0.1%) staining of membranes was performed to verify equal loading and transfer of proteins. Membranes were then incubated with antibodies to the p17 subunit of caspase-3 (1:200; Santa Cruz Biotech), procaspase-8 (1:1000; PharMingen), and caspase-9 (1:500; Calbiochem). Immune complexes were detected by subsequent incubation with appropriate horseradish peroxidase-conjugated antibodies to mouse or rabbit IgG (1:3000) and enhanced chemiluminescence (Pierce, Rockford, IL). Immunoblots were stripped of antibodies and reprobed with other antibodies to compare different proteins from the same blot as described in Rosenthal et al. (2003).

2.5. Bronchoalveolar lavage (BAL) collection from rats exposed to SM by inhalation

Male rats (250–300 g) were exposed to SM by inhalation, and bronchoalveolar lavage (BAL) samples were collected as described previously (Anderson et al., 1996). According to this procedure, anesthetized rats were intubated and subjected to SM inhalation by normal breathing. Twenty-four hours after SM exposure, bronchoalveolar lavage (BAL) samples were collected to analyze cellular components. Briefly, the rats were re-anesthetized and exsanguinated by clipping the vena cava. From each rat, the trachea was isolated and nicked, and a 16-gauge gavage needle was introduced and secured using a 3/0 suture. The lungs were lavaged with three 5-ml aliquots of saline as described in Anderson et al. (1996).

2.6. Statistical analysis

For caspase activity assays, data were compared using two-way ANOVA tests for significance: p values of <0.05 were considered statistically significant. The results are representative of at least three independent experiments with reproducible results.

3. Results

3.1. Caspase-8 is the pivotal caspase in both NHBE cells and SAEC

The central signaling proteins for many of the pathways that coordinate apoptosis are the caspases (cysteine proteases with aspartate at their substrate cleavage site), which cleave key proteins involved in the structure and integrity of the cell (Porter and Janicke, 1999; Kumar, 1999). To dissect the pathways of SM-induced apoptosis in airway epithelial cells, which may be responsible for the respiratory lesions, we determined whether caspase-8 was the pivotal caspase in the response of NHBE cells and SAEC to SM. Since caspase-3 has been shown to be a converging point for different apoptotic pathways, we first focused on caspase-3 proteolytic activation in the SM apoptotic response. Cells were exposed to SM at a concentration of either 100 or 300 μM in the absence or presence of inhibitors of caspase-8 or caspase-3 as described in Section 2. Proteolytic processing of procaspase-3 (p32) to the active form (p17) was noted in both SAEC (Fig. 1(A)) and NHBE cells (Fig. 1(B)) after exposure to SM in a concentration-dependent manner; this was consistent with our previous results and similar to keratinocyte responses to SM (Rosenthal et al., 2003). We previously observed that cultured human keratinocytes exposed to 100–300 μM SM undergo apoptosis as seen by caspase-3 activation and other apoptotic markers, e.g., nucleosome-sized DNA laddering, TUNEL-positive staining of cells and cleavage of the death substrate poly(ADP-ribose) polymerase (Rosenthal et al., 1998).

Next, we pretreated cells with a tetrapeptide inhibitor of caspase-8 or -3 prior to SM exposure. Fig. 1 shows that cells pretreated with the caspase-8 inhibitor almost completely failed to process caspase-3 to its active form. A very faint p17 band (Fig. 1(B), panel 7) was observed in NHBE cells, but none in SAEC (Fig. 1(A), panel 7). This is because NHBE cells contain a significantly higher level of caspase-9 compared to SAEC (Ray et al., 2008). These results are in agreement with those of caspase-3 activity in the presence of the caspase-8 inhibitor shown in Figs. 2(B) and 3(B). Taken together, these findings indicate that caspase-8 is the pivotal caspase in SM-exposed NHBE cells and SAEC, supporting the model of a DR pathway of apoptosis as we postulated (Ray et al., 2008). The effects of pretreating the cells with a caspase-3 inhibitor on caspase-3 processing and caspase-3 enzyme activity are shown in Figs. 1, 2(B) and 3(B), respectively. When cells were pretreated with a caspase-3 inhibitor, caspase-3 processing was inhibited, but not completely blocked; this would be expected if caspase-3 were to be processed by caspase-8. The bands shown in panel 5 of Fig. 1(A) and (B) do not represent the active form of caspase-3 as explained in Section 4; this is supported by the caspase-3 activity results (Figs. 2(B) and 3(B)).

3.2. Caspase-9 is activated by a feedback amplification loop in both NHBE cells and SAEC

To further understand the series of molecular events leading to apoptosis due to SM in airway epithelial cells, we studied the activation of key caspases, in particular the effector caspase-3, the upstream DR initiator caspase-8 and the upstream mitochondrial caspase-9. The results are shown in Figs. 2 and 3. Quantitative fluorometric caspase-3 and -8 activity assays using cytosolic extracts derived from SAEC and NHBE cells exposed to increasing doses of SM for 16 h revealed significant increases in both caspase-3 and -8 activity. These were similar to our previous observations in SM-exposed human epidermal keratinocytes (Rosenthal et al., 2003). Also, consistent with the results of proteolytic processing (Fig. 1), inhibition of caspase-8 completely blocked the activity of caspase-3 in SAEC and almost eliminated this activity in NHBE cells. Interestingly, inhibition of caspase-8 also greatly reduced the activation of caspase-9 in both SAEC and NHBE cells. Previously, it has been shown that caspase-8 can activate a mitochondrial pathway of apoptosis in certain cell types (Scaffidi et al., 1998). However, in the present case, caspase-9 appears to be activated *via* caspases-8 and -3, since inhibition of caspase-3 also blocks the activation of caspase-9. Caspase-3 inhibition blocked the activity of caspase-3 as expected and also blocked activation of caspase-8, although not to the same extent as it inhibited caspase-9. As explained later in Section 4, the blockade of caspase-8 due to caspase-3 inhibition does not imply a direct activation of caspase-8 by caspase-3.

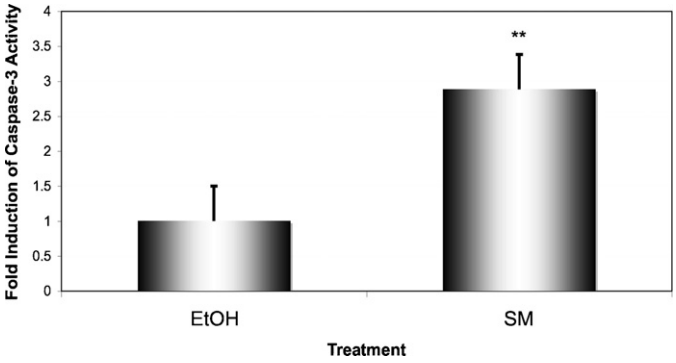

Fig. 4. SM-induced activation of caspase-3 in BAL fluid cells in the rat SM inhalation model. Rats were exposed to either ethanol (control) or SM by inhalation as described in Section 2. BAL fluid was collected at 24 h after SM exposure and centrifuged to obtain a cell pellet. Caspase-3 activity was assayed in cytosolic extracts. Error bars represent average ± SEM, $n = 3$. Significance relates to untreated control (0 μM SM) as follows, $^{**}p < 0.01$.

3.3. SM induces caspase-3 in vivo

To determine if SM induces apoptosis *in vivo*, rats were exposed to SM by inhalation by intubation as described in Section 2, bronchoalveolar lavage (BAL) samples were collected and cells in BAL samples were subjected to fluorometric assays for caspase-3 activity. Results presented in Fig. 4 show that caspase-3 was significantly ($p < 0.05$) activated *in vivo* following SM inhalation exposure. This observation correlates with the observed sloughing of the tracheal and bronchial epithelia at the mucosal/submucosal interface in rats similarly exposed to SM by inhalation (Anderson et al., 1996). In this study, histological examination of the detached epithelium revealed scattered individual and small aggregates of cells that had become condensed and intensely eosinophilic with pyknotic or karyorrhectic nuclei; these are typical morphological expressions of apoptotic cell death.

4. Discussion

Previously, we reported that SM induces apoptosis in cultured normal human airway epithelial cells predominantly *via* the DR pathway (Ray et al., 2008). We also showed that NHBE cells are exquisitely sensitive to SM relative to the SAEC; this is consistent with the greater degree of damage observed in bronchi vs. alveoli in individuals exposed to SM (Balali-Mood and Hefazi, 2006). In this study, we show that in apoptosis due to SM, a feedback amplification loop appears to activate caspase-9 *via* caspase-3. Hypothetical schemes of this amplification loop (indicated by thick arrows) are presented in Fig. 5. According to these schemes, SM induces apoptosis by both the intrinsic mitochondrial pathway and the extrinsic DR (Fas/Fas ligand) pathway (Rosenthal et al., 1998, 2003). Caspase-8 is the initiator caspase in the DR pathway, whereas caspase-9 is the initiator caspase in the intrinsic pathway. Both pathways activate caspase-3 which is the executioner caspase responsible for the end effects in the apoptosis process. We examined these three key

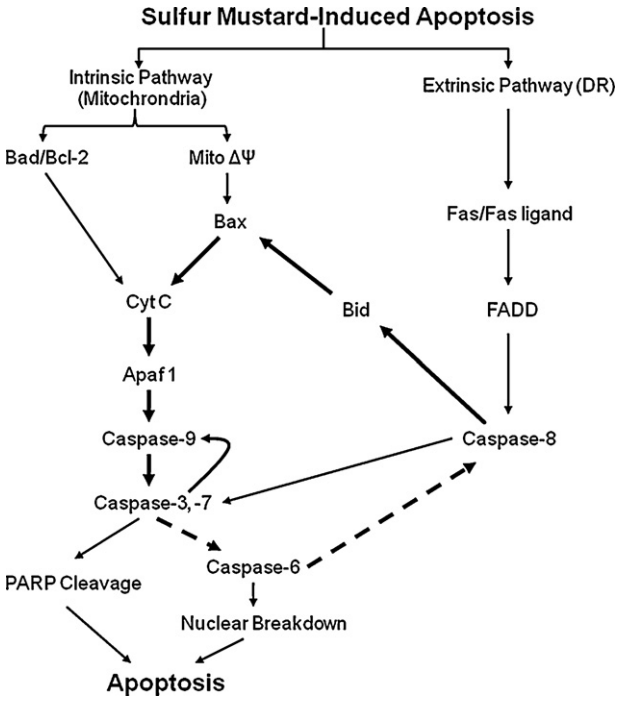

Fig. 5. Hypothetical scheme of an amplification loop between caspases-8, -9, -3 and -6 contributing to SM-induced caspase-8 exacerbation in normal human airway epithelial cells. Dotted lines indicate data not shown.

caspases, -8, -9 and -3, to establish their roles in SM-induced apoptosis in NHBE cells and SAEC to include the proposed amplification loop.

As explained in Section 3, caspase-8 is the pivotal enzyme in SM-induced apoptosis. Caspase-8 is also the initiator enzyme in the amplification loop; moreover, caspase-8 is stimulated by caspase-3 *via* caspase-6 (Cowling and Downward, 2002). We used the inhibitors of caspases-8 and -3 to elucidate further their roles in the amplification loop. According to the proposed amplification scheme, inhibiting caspase-3 activity would block the amplification steps *via* caspase-3, but both caspases-8 and -9 would be activated *via* the extrinsic and the intrinsic pathways, respectively. The results presented in Figs. 2 and 3 show that the caspase-3 inhibitor blocks caspase-3 itself, as expected; however, there is still some stimulation of caspase-8, but not of -9. The absence of caspase-9 activity can be explained as due to the elimination of the positive feedback loop in which active caspase-3 processes procaspase-9 into its fully active form (Fujita et al., 2001). Thus in human airway epithelial cells, caspase-8 appears to be the pivotal caspase for activation of caspase-3, which in turn activates both caspases-8 and -9. By a similar analysis, inhibiting caspase-8 activity would block the extrinsic pathway itself as well as the amplification steps *via* caspase-8. Our results (Figs. 2 and 3) are in agreement with this analysis; both caspases-8 and -9 are blocked. The residual activity of caspase-3 in NHBE cells (Fig. 3(B)) may in fact be due to trace a amount of caspase-9 activity and/or an additional (e.g., cathepsin D-dependent) pathway (Trincheri et al., 2007).

The critical event in the proposed amplification loop is the caspase-8-dependent activation of caspase-9, *via* caspase-3. Caspase-8 can activate caspase-9 *via* proteolysis of Bid, which stimulates the intrinsic mitochondrial pathway of apoptosis by enhancing cytochrome c release. When the amplification loop is blocked, e.g., by a caspase-3 inhibitor, there is still a minor processing of caspases-3 (Fig. 1); however, the weak caspase-9 response is insufficient to cause complete caspase-3 processing and activation. On the other hand, inhibiting caspase-8 virtually eliminates caspase-3 processing (Fig. 1) as well as caspase-9 activation (Fig. 3(C)). We postulate that in the presence of caspase-3 inhibitor, P20/P12 (weakly active caspase-3 intermediate) is the predominant form as opposed to P17/P12 (fully active caspase-3) seen in cells treated with SM alone (Fig. 1(B), lane 5 vs. 3) *via* involvement of XIAP and Smac/Diablo (Srinivasula et al., 2001; Sun et al., 2002; Bratton and Cohen, 2003) The involvement of Bid as shown in Fig. 5 is suggested by the effects of caspase-3 or -8 inhibitors. The lack of caspase-3 processing when caspase-8 is inhibited can be explained if Bid is involved in caspase-9 processing and activation by caspase-8. The partial processing of caspase-3 observed (Fig. 1) when caspase-3 is inhibited can also be explained by the Bid mediated caspase-9 processing and activation *via* caspase-8 (Fulda et al., 2001). While the relative contributions of these feedback amplification loops are hard to determine, at least one other study has shown that caspase-9 can be activated *via* caspases-3 by a process involving cleavage and elimination of an XIAP inhibitory binding site (Zou et al., 2003). Whether this is occurring in SM-treated NHBE cells or SAEC requires further studies.

There are reports using human bronchial epithelial cells suggesting intervention of SM toxicity by preventing the mitochondrial pathway of apoptosis (Sourdeval et al., 2006) and other cytotoxic mechanisms (Rappeneau et al., 2000). Recently, Kehe et al. (2009) have extensively reviewed the molecular toxicology and therapy of skin toxicity due to SM. In our study, we have obtained new information on the role of the mitochondrial mechanism in the caspase amplification cascade due to SM. Our results have also explained how the mitochondrial mechanism could be a basis for the difference in SM sensitivity between SAEC and NHBE cells. Additionally, we have shown that lungs exposed to SM by inhalation undergo a caspase-3 apoptotic response *in vivo* in rats. This provides justification for further *in vivo* studies utilizing the suppression of the DR response by either chemical inhibitors or a molecular method, e.g., RNA interference for therapeutic development.

Conflict of interest

None.

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